Glycobiology, 2022, **32**, 9, 736–742
https://doi.org/10.1093/glycob/cwac043
Advance access publication date 5 July 2022
GlycoForum - Technical Note

The incorrect use of CD75 as a synonym for ST6GAL1 has fostered the expansion of commercial "ST6GAL1" antibodies that do not recognize ST6GAL1

Barnita Haldar[1], Jihye Hwang[1], Yoshiki Narimatsu[2], Henrik Clausen[2], Susan L Bellis[1,*]

[1]Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA , [2]Department of Cellular and Molecular Medicine, Copenhagen Center for Glycomics, University of Copenhagen, Copenhagen, DK-1165, Denmark

*Corresponding author: Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, MCLM 350, 1918 University Boulevard, Birmingham, AL 35294, USA. Email: bellis@uab.edu

The ST6GAL1 Golgi sialyltransferase is upregulated in many human malignancies, however, detection of ST6GAL1 protein in cancer tissues has been hindered by the prior lack of antibodies. Recently, numerous commercial antibodies for ST6GAL1 have become available, however, many of these do not, in fact, recognize ST6GAL1. Decades ago, the CD75 cell-surface epitope was mistakenly suggested to be the same molecule as ST6GAL1. While this was rapidly disproven, the use of CD75 as a synonym for ST6GAL1 has persisted, particularly by companies selling "ST6GAL1" antibodies. CD75 is reportedly a sialylated epitope which appears to encompass a range of glycan structures and glycan carriers. In this study, we evaluated the LN1 and ZB55 monoclonal antibodies, which are advertised as ST6GAL1 antibodies but were initially developed as CD75-recognizing antibodies (neither was raised against ST6GAL1 as the immunogen). Importantly, the LN1 and ZB55 antibodies have been widely used by investigators, as well as the Human Protein Atlas database, to characterize ST6GAL1 expression. Herein, we used cell and mouse models with controlled expression of ST6GAL1 to compare LN1 and ZB55 with an extensively validated polyclonal antibody to ST6GAL1. We find that LN1 and ZB55 do not recognize ST6GAL1, and furthermore, these 2 antibodies recognize different targets. Additionally, we utilized the well-validated ST6GAL1 antibody to determine that ST6GAL1 is overexpressed in bladder cancer, a finding that contradicts prior studies which employed LN1 to suggest ST6GAL1 is downregulated in bladder cancer. Collectively, our studies underscore the need for careful validation of antibodies purported to recognize ST6GAL1.

Key words: cancer; CD75; CDw75; sialic acid; ST6GAL1.

Introduction

Aberrant glycosylation, including increased cell-surface sialylation, is an important biomarker of a tumor cell (Pinho and Reis 2015; Bellis et al. 2022). One prevalent sialoglycan enriched in tumor cells is the α2–6-linked sialic acid modification added to galactose on *N*-glycans. In most tissues, this modification is elaborated primarily by the ST6GAL1 Golgi sialyltransferase. The ST6GAL1 enzyme is upregulated in numerous human malignancies and a functional role for ST6GAL1 in promoting carcinogenesis is well established (Lu and Gu 2015; Garnham et al. 2019; Dorsett et al. 2021).

Historically, the availability of specific antibodies for ST6GAL1 has been limited, which, in turn, has hindered an assessment of ST6GAL1 protein expression in both normal and malignant tissues. However, increasing interest in tumor sialylation has recently prompted a marked expansion in commercial sources of antibodies claimed to react with ST6GAL1. Unfortunately, many of these antibodies do not recognize ST6GAL1 protein, and in fact, many were not even raised against ST6GAL1 as the immunogen. In a single study published in 1990 (Stamenkovic et al. 1990), ST6GAL1 was suggested to be the same molecule as the cell-surface glycan epitope, CD75, also known as CDw75 or CD75s. This was firmly disproven in 1992 (Bast et al. 1992; Keppler et al. 1992; Munro et al. 1992), and an extensive literature since that time has confirmed that ST6GAL1 is a Golgi-localized protein and not CD75. However, the terms, ST6GAL1, CD75, CDw75, and CD75s are currently being used synonymously by many companies offering "ST6GAL1" antibodies. Many of these "ST6GAL1" antibodies actually recognize CD75-related glycan epitopes and not ST6GAL1.

CD75 antibodies comprise a large and diverse group of monoclonal antibodies (mAbs) generated many years ago. The glycan epitope recognized by CD75 is generally enriched in immune cells, particularly B lymphocytes (Guy and Andrew 1991; Bast et al. 1992). Sialic acid is reported to be a component of the epitope, although evidence suggests the various CD75 mAbs detect distinct antigens (Guy and Andrew 1991; Bast et al. 1992). It remains unclear whether this disparity in antigen recognition is due to variations in the glycan structure and/or in the carrier proteins. Moreover, some CD75 glycan epitopes, particularly those recognized by CD75s mAbs, are present on gangliosides rather than glycoproteins (Souady et al. 2011). The critical requirement for sialic acid as part of the CD75 epitope was inferred, in part, because antibody binding was reduced by pretreating cells with a neuraminidase enzyme that cleaves α2–3-, α2–6-, and α2–8-linked sialic acids (Epstein et al. 1984; Guy and Andrew 1991; Bast et al. 1992). Additionally, several of the CD75 antibodies have increased binding to cells engineered with ST6GAL1 overexpression (Bast et al. 1992; Keppler et al. 1992). However, it is not clear that the CD75 antibodies have a restricted specificity for α2–6-linked sialic acids, as opposed to recognizing sialic acid independent of the type of linkage. Importantly, diverse immunogens were used to generate the family of CD75 antibodies, although most immunogens were from immune populations, including myeloid, lymphoid, and B lymphoma cells. Given this diversity of immunogens, it is

Received: June 2, 2022. **Revised:** June 2, 2022. **Accepted:** June 27, 2022

not surprising that the various CD75 antibodies appear to recognize distinct targets. Examples of widely used CD75 antibodies include the LN1 mAb clone (Epstein et al. 1984), which was generated against a nuclear homogenate from pokeweed mitogen-stimulated lymphocytes (of note, there is no evidence that ST6GAL1 is ever found in the nucleus), and the ZB55 mAb clone, which was raised against a peptide from the cell-surface immune receptor, FcγRIII (CD32) (Pulford 1996). Both the LN1 and ZB55 mAbs are advertised by commercial sources as ST6GAL1 antibodies.

Notably, the LN1 and ZB55 mAbs were employed by the Human Protein Atlas database to characterize the expression of the ST6GAL1 enzyme in human tissues. The Human Protein Atlas has emerged as a major resource for assessing protein levels of various target molecules, therefore, flawed results could cause much confusion in the field. Furthermore, the LN1 antibody has been used to monitor ST6GAL1 expression in human cancers (Antony et al. 2014), and more recently, the epitope for LN1 was identified as an important marker for naive pluripotent stem cells (Collier et al. 2017). To address the discrepancy regarding CD75 and ST6GAL1 antibodies, we conducted a variety of assays on cells and tissues with controlled ST6GAL1 expression to directly compare the LN1 and ZB55 mAbs with an anti-ST6GAL1 polyclonal antibody (pAb) that was raised against recombinant ST6GAL1 protein and has been extensively confirmed to bind ST6GAL1 (Swindall et al. 2013; Schultz et al. 2016; Dorsett et al. 2021). Here, we show that the LN1 and ZB55 mAbs do not bind STGAL1, and in fact, they even appear to recognize different targets. Additionally, we conducted immunohistochemistry (IHC) on normal and malignant human bladder tissues using the well-validated ST6GAL1 antibody and found that ST6GAL1 is overexpressed in bladder cancer. This finding contradicts prior studies that employed LN1 to suggest that ST6GAL1 is downregulated in bladder cancer (Antony et al. 2014). These collective studies underscore the need to examine the immunogen used to generate commercial "ST6GAL1" antibodies and for careful validation of such antibodies.

Results and discussion

The LN1 and ZB55 clones do not recognize ST6GAL1, as indicated by immunocytochemistry conducted on cells with modulated ST6GAL1 expression

To test antibody specificity, we used HEK293 cell lines with genetic deletion of either the α2–3 sialyltransferases, ST3GAL3, ST3GAL4, and ST3GAL6 (ΔST3), or the α2–6 sialyltransferases, ST6GAL1 and ST6GAL2 (ΔST6) (Narimatsu et al. 2019). Additionally, to act as a rescue model, ST6GAL1 was reexpressed in the ΔST6 line (ΔST6-R). Immunofluorescent staining using a validated ST6GAL1 pAb from R&D Systems revealed ST6GAL1 expression in WT and ΔST3 cells but not in ΔST6 cells (Fig. 1A, top panels). Staining for ST6GAL1 was restored in the ΔST6-R cell line. The R&D pAb has been extensively validated using a wide range of cell lines with modulated ST6GAL1 expression as well as tissues from genetically engineered mice with ST6GAL1 overexpression or knock-out (Swindall et al. 2013; Schultz et al. 2016; Dorsett et al. 2021). Staining by the R&D antibody presented as a punctate pattern adjacent to the nucleus, in agreement with studies of many other Golgi-localized glycosyltransferases (Steentoft et al. 2019). Golgi localization of ST6GAL1 was confirmed by costaining for the Golgi protein, GM-130 (Supplementary Fig. S1A).

The HEK293 cell lines were then stained with the ZB55 and LN1 antibodies (Fig. 1A, middle and lower panels, respectively). The ZB55 antibody stained all 4 of the cell lines similarly, and importantly, ZB55 binding was not abrogated by the loss of ST6GAL1 expression. The staining pattern for the ZB55 clone was diffuse and cytosolic rather than Golgi-localized. In contrast to ZB55, the LN1 antibody failed to react with any of the HEK293 lines including cells with overexpressed ST6GAL1 (ΔST6-R).

Results were confirmed using a second cell model, human Suit2 pancreatic cancer cells. Although most cancer cell lines have robust ST6GAL1 expression, Suit2 cells are unusual in that they have very low levels of endogenous ST6GAL1 (Britain et al. 2021). We therefore generated a Suit2 cell line with overexpression of ST6GAL1 (OE), along with an empty vector (EV) control, as previously described (Britain et al. 2021). Strong staining for ST6GAL1 was observed in OE, but not EV, cells using the R&D antibody (Fig. 1B). The ZB55 antibody stained the Suit2 EV and OE lines at equivalent levels, whereas no staining was detected in either of the lines by LN1.

Flow cytometry analyses show that the ZB55 antibody, but not LN1 or the validated ST6GAL1 antibody, recognizes a cell-surface epitope

Antibody specificity was next evaluated by flow cytometric analyses conducted with nonpermeabilized cells, given that CD75 is reported to be a cell-surface epitope. We used the CA-46 B lymphoma cell line as a model because CD75 is enriched in B cells, and furthermore, B cells express robust levels of both ST6GAL1 and α2–6 sialic acid-capped surface glycans. No detectable staining of CA-46 cells was observed with the R&D ST6GAL1 antibody, consistent with the fact that ST6GAL1 is localized to the Golgi (Fig. 1C). Interestingly, the ZB55, but not LN1, antibody strongly stained the surface of CA-46 cells. To determine whether ZB55 recognition was affected by the level of α2–6 sialylation, cells were treated with either neuraminidase S, which specifically cleaves α2–3-linked sialic acids, or with the *Arthrobacter ureafaciens* neuraminidase, which prefers α2–6-linked sialic acids but can also cleave α2–3 and α2–8 sialic acid linkages. As shown in Fig. 1E, levels of staining by the ZB55 antibody were slightly reduced by both neuraminidases. As well, the 2 neuraminidases had an equivalent effect on ZB55 binding, suggesting that the epitope bound by ZB55 does not require the α2–6 sialic acid linkage for recognition. We also examined ZB55 binding to Suit2 EV and OE cells. The overall levels of ZB55 staining were very low for EV and OE cells and no differences were noted in staining intensity (Supplementary Fig. S1B). As mentioned previously, the immunogen used to generate the ZB55 antibody was a peptide derived from FcγRIII, which is abundant in immune cells but not typically found on epithelial cells. Pretreatment with the *A. ureafaciens* neuraminidase slightly reduced staining in both EV and OE cells (Supplementary Fig. S1C). Together, the flow cytometry data reinforce the immunocytochemistry results (Fig. 1A and B) suggesting that the LN1 and ZB55 antibodies recognize different targets, neither of which is ST6GAL1.

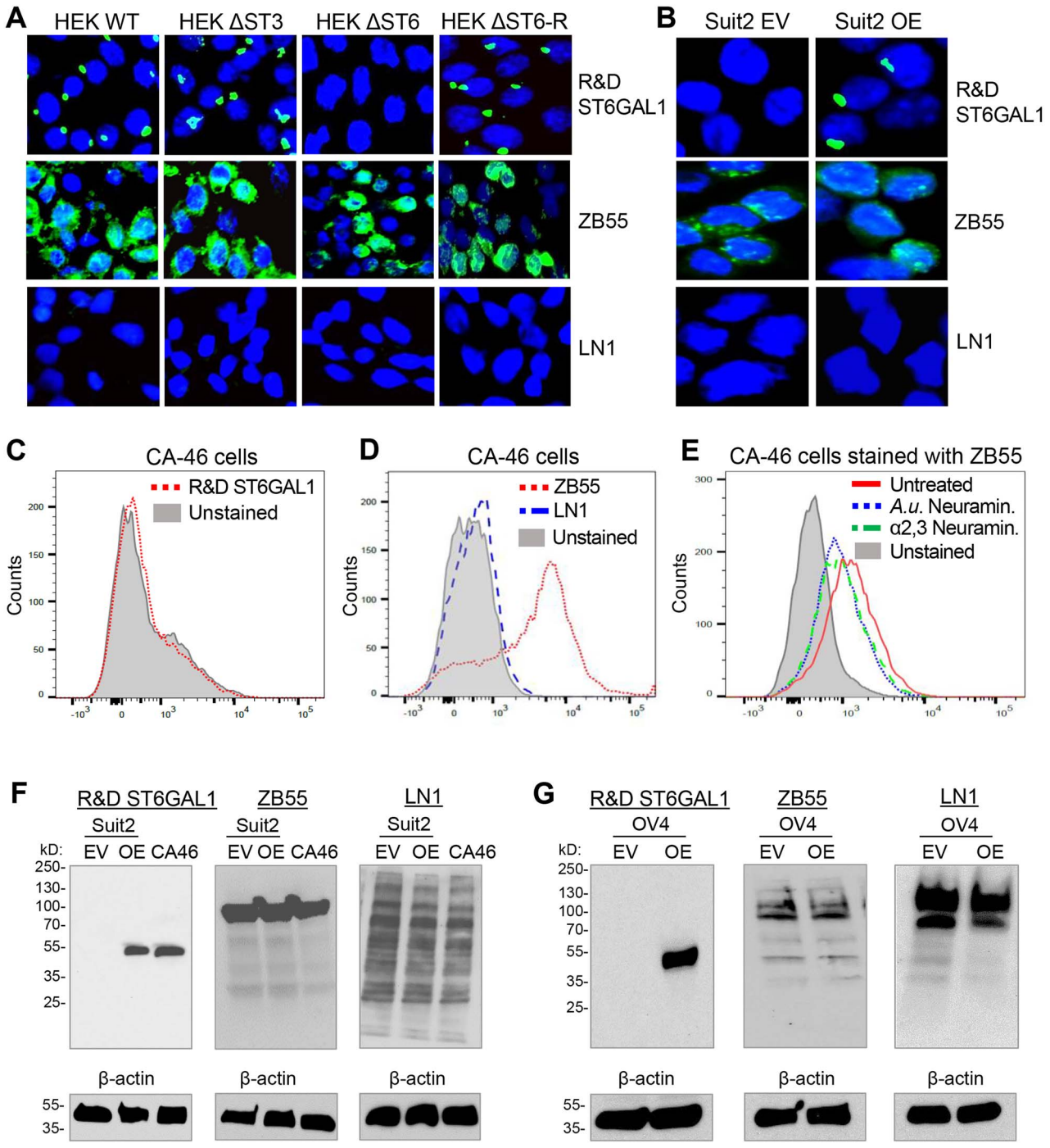

Fig. 1. The validated R&D ST6GAL1 antibody, but not the ZB55 or LN1 clones, recognizes ST6GAL1 in cell models with modulated ST6GAL1 expression. A) Immunocytochemistry was conducted on WT HEK 293 cells or HEK 293 cells with CRISPR/Cas9-mediated deletion of select glycosyltransferases. The ΔST3 cell line has deletions in the 3 main sialyltransferases that add α2–3-linked sialic acid to *N*-glycans, namely ST3GAL3, ST3GAL4, and ST3GAL6. The ΔST6 cell line has deletions in the 2 sialyltransferases that add α2–6-linked sialic acid to *N*-glycans, ST6GAL1 and ST6GAL2. For the ΔST6-R cell line, ST6GAL1 was reexpressed in the ΔST6 cell line. Cells were stained with the R&D ST6GAL1 pAb, and the ZB55 and LN1 mAbs. B) Suit2 pancreatic cancer cells with ST6GAL1 overexpression (OE) or EV control cells were stained with the R&D ST6GAL1, ZB55, and LN1, antibodies. C) Flow cytometry experiments with nonpermeabilized cells show that the R&D antibody fails to detect a surface epitope on CA-46 B cells. D) The ZB55 antibody, but not LN1, detects a surface epitope on CA-46 cells. E) ZB55 binding to CA-46 cells is reduced by pretreatment with either neuraminidase S, which cleaves α2–3-linked sialic acids, or with *A. ureafaciens* (*A.u.*) neuraminidase, which preferentially cleaves α2–6 sialic acids but can also cleave α2–3 and α2–8 sialic acid linkages. F) The R&D ST6GAL1 antibody detects a ~50 kD band in immunoblots of Suit2 OE, and CA-46, cell lysates, but not Suit2 EV lysates. Immunoblotting with the ZB55 and LN1 antibodies yields multiple, nonspecific bands in all cell lines. G) The R&D ST6GAL1 antibody detects a ~50 kD band in OV4 OE, but not EV, cell lysates. Immunoblotting with the ZB55 and LN1 antibodies yields multiple, nonspecific bands in all cell lines.

The validated ST6GAL1 antibody, but not ZB55 or LN1, recognizes ST6GAL1 in immunoblotting experiments

Whole cell lysates from Suit2 EV and OE cells, as well as CA-46 cells, were immunoblotted using the 3 antibodies. Probing with the R&D ST6GAL1 pAb revealed a single band at ~50 kD in Suit2 OE and CA-46 cells (Fig. 1F), consistent with the expected size of the full-length ST6GAL1 protein (Weinstein et al. 1987). However, no detectable bands were observed in Suit2 EV lysates. In stark contrast, multiple bands were observed in all 3 cell lines when immunoblotting with the ZB55 and LN1 mAbs. Immunoblotting was also performed with OV4 ovarian cancer cells, another unusual cancer cell line that lacks detectable endogenous ST6GAL1 protein expression. As with Suit2 cells, the R&D ST6GAL1 antibody recognized a ~50 kD protein in OV4 OE, but not EV, cells, whereas the ZB55 and LN1 antibodies bound to multiple, nonspecific bands (Fig. 1G). Immunoblotting is an important method for antibody testing because the size of the target molecule can be verified (Uhlen et al. 2016). However, protein denaturation disrupts discontinuous epitopes that may be important for antibody recognition. Immunocytochemistry, a nondenaturing approach, preserves these epitopes. Our immunoblotting results, combined with immunocytochemistry conducted on ST6GAL1 knockout cells (Fig. 1A), provide strong evidence that the R&D pAb specifically recognizes ST6GAL1 and does not cross-react with other proteins.

IHC staining with the validated ST6GAL1 antibody, but not LN1 or ZB55, detects ST6GAL1 in tissues from mice with transgenic expression of ST6GAL1

IHC analyses are important for delineating cell type-specific expression of a target protein, relative protein levels, and aberrant expression of protein in diseased tissues. We thus conducted IHC staining using tissues from mice with transgenic expression of human ST6GAL1 (*Rosa26-LSL-ST6GAL1*), generated as previously described (Schultz et al. 2016). This line was crossed to the Pdx1-Cre line to drive ST6GAL1 expression in the pancreas. As shown in Fig. 2A, staining with the R&D ST6GAL1 antibody revealed robust ST6GAL1 expression in the pancreatic acinar cells of transgenic mice, whereas acinar cells from WT mice lacked ST6GAL1 expression (of note, the R&D antibody does recognize murine ST6GAL1 by IHC, but acinar cells have undetectable levels of endogenous ST6GAL1). On the other hand, no detectable staining was observed with the ZB55 and LN1 antibodies in the acinar cells of either WT or ST6GAL1 transgenic mice. (Fig. 2B and C). Interestingly, positive staining by ZB55 and LN1 was detected in cells within the blood vessels of the pancreas (Supplementary Fig. S2A and B), suggesting that these antibodies have selectivity for some epitope on immune cells.

ST6GAL1 expression is upregulated in human bladder cancer

Prior IHC studies using the LN1 antibody suggested that ST6GAL1 expression is downregulated in bladder cancer, which runs counter to an extensive literature suggesting that ST6GAL1 is typically upregulated in epithelial cancers. Accordingly, we conducted IHC staining on tissue microarrays containing normal and malignant human bladder specimens. While normal bladder surface epithelium was not stained by the R&D ST6GAL1 pAb, bladder cancer tissue sections harbored numerous ST6GAL1-positive cancer cells (Fig. 2D). The staining pattern appeared as multiple punctae, which is characteristic of the known disorganization of the Golgi in cancer cells (Kellokumpu et al. 2002). Quantification of ST6GAL1-positive cells confirmed an increase in ST6GAL1 expression across all stages of bladder cancer, with significantly higher levels noted in stage III compared with stage I malignancies (Fig. 2E). Conversely, the ZB55 and LN1 antibodies failed to detect ST6GAL1 in bladder cancer cells (Fig. 2F and G), although both mAbs showed reactive cells within the blood vessels of cancer specimens (Supplementary Fig. S2C).

Conclusions

Many commercial antibodies advertised as "ST6GAL1" antibodies were originally developed to recognize the CD75 surface epitope, which was mistakenly interpreted as the ST6GAL1 enzyme. Unfortunately, these antibodies have been widely used to evaluate ST6GAL1 expression, leading to confusion regarding the levels of ST6GAL1 expression in specific cell types and tissues as well as changes in ST6GAL1 expression during pathogenesis. In the current study, we compared 2 such CD75 antibodies, ZB55 and LN1, with a well-validated anti-ST6GAL1 antibody. Our collective results show that: (i) ZB55 and LN1 do not recognize ST6GAL1, (ii) ZB55 and LN1 detect different target molecules, (iii) ST6GAL1 activity is not essential for generating the epitopes recognized by ZB55 and LN1, and (iv) the R&D pAb is a reliable reagent for detecting ST6GAL1.

Materials and methods

Cell culture

HEK293 cell lines with CRISPR/Cas9-mediated deletion of combinations of *ST3GAL* genes (*ST3GAL3*, *ST3GAL4* and *ST3GAL6*, designated as ΔST3) or *ST6GAL* genes (*ST6GAL1* and *ST6GAL2*, designated as ΔST6) were generated and characterized as previously described (Narimatsu et al. 2019). ST6GAL1 was reexpressed in the ΔST6 line (designated as ΔST6-R) using lentivirus (GeneCopoeia, see Supplementary Table S1 for reagent information). Cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS, 1% antibiotic/antimycotic, and 1% glutamax solution (Thermo Fisher). CA-46 cells were purchased from ATCC and cultured in RPMI medium with 20% FBS and 1% antibiotic/antimycotic solution. Suit2 and OV4 cell lines with ST6GAL1 overexpression were generated and maintained as previously described (Britain et al. 2017, 2021).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 10 min. Cells were blocked with 10% horse serum for 1 h and incubated with the following primary antibodies at 4 °C overnight: anti-ST6GAL1 (R&D Systems, AF5924), ZB55 (Santa Cruz, sc-20,063), and LN1 (Santa Cruz, sc-6263) (see Supplementary Table S1). Cells were then incubated with the appropriate secondary antibody and counterstained with Hoescht. After staining, coverslips were mounted on slides using Prolong Gold (Thermo Fisher) mounting medium.

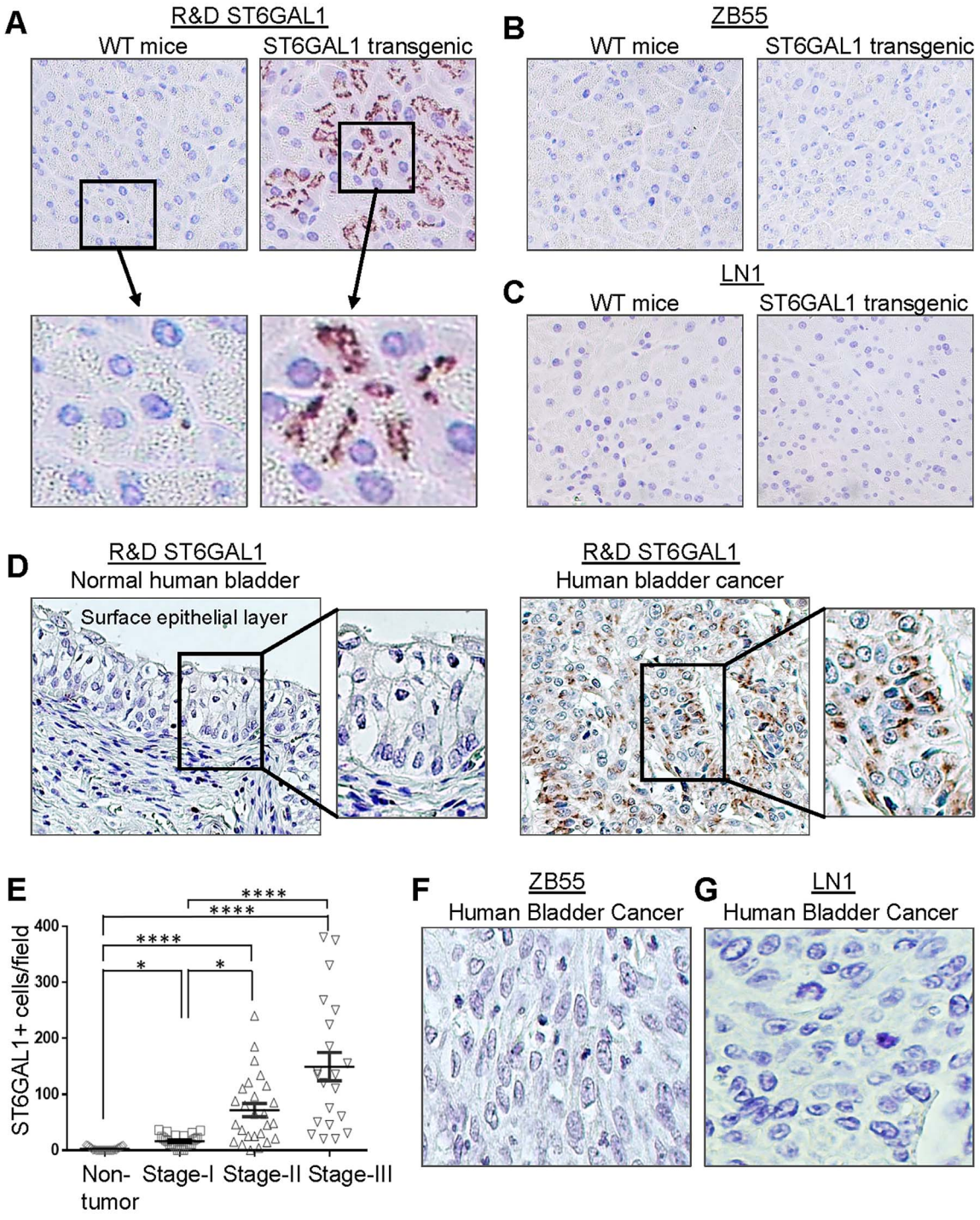

Fig. 2. The R&D ST6GAL1 antibody, but not ZB55 or LN1, recognizes ST6GAL1 in tissues from mice with transgenic expression of ST6GAL1 transgene, and also in human bladder cancer cells. A) IHC staining with the R&D ST6GAL1 antibody detects ST6GAL1 in the acinar cells of transgenic mice expressing human ST6GAL in the pancreas. No staining is noted in WT acinar cells, which are known to have undetectable levels of ST6GAL1. B) The ZB55 antibody does not detect ST6GAL1 in transgenic mice. C) The LN1 antibody does not detect ST6GAL1 in transgenic mice. D) Tissue microarrays containing normal and malignant human bladder tissues were IHC stained with the R&D ST6GAL1 antibody. ST6GAL1 was detected in bladder cancer cells but not in the normal bladder epithelium. E) ST6GAL1-positive cancer cells stained with the R&D antibody were quantified across varying stages of bladder cancer. $^{*}P < 0.05$; $^{****}P < 0.0001$. F) No detectable staining of bladder cancer cells was observed with the ZB55 antibody. G) No detectable staining of bladder cancer cells was observed with the LN1 antibody.

Flow cytometry

Nonpermeabilized cells were stained with ZB55, LN1, or the R&D ST6GAL1 pAb. For some experiments, cells were pretreated for 60 min with either *A. ureafaciens* neuraminidase (Sigma, #10269611001) or α2–3-specific neuraminidase S (BioLabs, #P0743L). Cells were analyzed using an LSRII flow cytometer and FlowJo V10 was used for data analysis.

Immunoblotting

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (ThermoFisher). BCA assay (Pierce) was used for protein quantification. PVDF membranes were blocked in 5% nonfat dry milk for 1 h in Tris-buffered saline with 0.1% Tween 20. Blots were incubated with primary antibodies overnight at 4 °C, followed by a 1-h incubation at room temperature with appropriate secondary antibodies. Membranes were developed using Clarity Western ECL substrate (BioRad) or SuperSignal West Femto substrate (Pierce).

Immunohistochemistry

IHC was conducted on tissue microarrays containing normal and malignant human bladder specimens (Biomax Inc., TMA# BL1002b, BL481b, T121b, and BL244b) as well as pancreatic tissues from mice with transgenic expression of ST6GAL1 (Schultz et al. 2016). Using an established IHC protocol (Schultz et al. 2016), tissue sections were subjected to antigen retrieval with Antigen Unmasking Solution, Citric Acid Based (Vector Labs) and then blocked with 2.5% horse serum for 1 h. Sections were incubated with primary antibodies at 4 °C overnight, followed by appropriate secondary antibodies (Immpress, Vector Labs) for 1 h at room temperature. Reactions were developed using ImmPACT NovaRED Peroxidase substrate or ImmPACT DAB EqV Peroxidase Substrate. Sections were counterstained with hematoxylin. Slides were mounted using VectaMount mounting medium (Vector Laboratories) and images were captured using a Nikon 80i Eclipse microscope. Images were processed with NIS Elements Imaging Software. Statistical analysis of ST6GAL1 expression in patient samples was performed in GraphPad Prism using the nonparametric Kruskal-Wallis ANOVA test, followed by Dunn's multiple comparison test. A nonparametric test was selected after assessing the data with the D'Agostino-Pearson normality test.

Acknowledgements

The authors gratefully acknowledge assistance from the UAB Flow Cytometry Core Facility.

Supplementary material

Supplementary material is available at *Glycobiology Journal* online.

Funding

This work was supported by the National Institutes of Health (CA225177 and CA233581 to S.L.B.) and the Danish National Research Foundation (DNRF107 to H.C.).
Conflict of interest statement. None declared.

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